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PHEROMONAL ACTIVITY OF COMPOUNDS IDENTIFIED FROM MALE *Phyllotreta cruciferae*: FIELD TESTS OF RACEMIC MIXTURES, PURE ENANTIOMERS, AND COMBINATIONS WITH ALLYL ISOTHIOCYANATE

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Abstract—Four himachalene sesquiterpenes and (+)-γ-cadinene, previously identified as possible pheromone components from males of a North American population of *Phyllotreta cruciferae* Goeze (Coleoptera, Chrysomelidae), were tested for attractiveness in field trapping experiments in Hungary. A mixture of the four synthetic racemic himachalene derivatives and (+)-γ-cadinene from a botanical source was slightly attractive to beetles, but much more attractive when blended with the known host-plant-derived attractant allyl isothiocyanate. This result was consistent with a previous study in North America. In tests with optically pure synthetic compounds, a blend of the same himachalene enantiomers found from male beetles was equivalent to the corresponding blend of racemic compounds, whereas a blend of the opposite enantiomers was not active. Through subtraction tests, it was found that the single compound, (6*R*,7*S*)-2,2,6,10-tetramethylbicyclo[5.4.0.]-undeca-9,11-diene [compound (+)-**A** in this study], was as active as the whole mixture, suggesting that this compound is the key pheromone component of the European population of *P. cruciferae*. During field trials, several

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congeneric species, including *P. vittula*, *P. nemorum*, *P. nodicornis*, and *P. ochripes*, also were caught, suggesting that the same compound(s) may be relatively widespread as pheromone components in this genus.

Key Words V *Phyllotreta cruciferae*, *Phyllotreta* spp., Coleoptera, Chrysomelidae, field trapping, pheromone, sesquiterpene, himachalene, cadinene, enantiomer, electrophysiology

INTRODUCTION

Flea beetles of the genus *Phyllotreta* (Coleoptera, Chrysomelidae, Halticinae) are important pests of cruciferous crops such as cabbage, rapeseed, and radishes, both in Europe (Jourdheuil, 1966; Sáringer, 1998) and in North America (Lamb, 1989). Their importance as pests is aggravated by the fact that several species are known to vector plant pathogens (Markham and Smith, 1949; Campbell and Colt, 1967; Ryden, 1989; Dillard et al., 1998; Glits, 2000).

Effective tools to detect and monitor flea beetles would be of great utility in their control. The secondary plant metabolite allyl isothiocyanate (allyl ITCN) has long been known as a feeding and oviposition stimulant and also as an attractant for *P. cruciferae* Goeze and other *Phyllotreta* spp. (Görnitz, 1956; Feeny et al., 1970; Hicks, 1974; Vincent and Stewart, 1984; Pivnick et al., 1992).

Male *P. cruciferae* produce an aggregation pheromone (Peng and Weiss, 1992; Peng et al., 1999). Analysis of volatiles emitted by males of a North American population of *P. cruciferae* identified six sesquiterpenes (Figure 1) as candidate pheromone components (Bartelt et al., 2001). These were produced only by males, although the major compound (**A**) was readily sensed by the antennae of both sexes (Bartelt et al., 2001). These compounds, or a subset of them, was thought likely to constitute the pheromone. All six compounds are chiral, and only the enantiomers shown in Figure 1 are emitted by the beetles. The beetle-derived enantiomers all have a positive optical rotation in hexane (Bartelt et al., 2001), and signs of rotation used below refer to dilute hexane solutions.

Assignment of the true stereochemistry for **A**, **C**, **E**, and **H** has been complicated. The configurations were initially determined by Bartelt et al. (2001): relative stereochemistry of the two stereogenic centers of **A**, **C**, and **H** was based on NMR and molecular modeling. Assignment of their absolute configurations was based on linking them to **E** through chemical conversions and comparison of the optical rotation of **E** to literature information. Pandy and Dev (1968) had reported an enantiomer of **E** from Himalayan cedar trees and established its absolute configuration as (*S*) by enantioselective synthesis. Beetle-derived **E** had a specific rotation that was of the same sign (+) as that

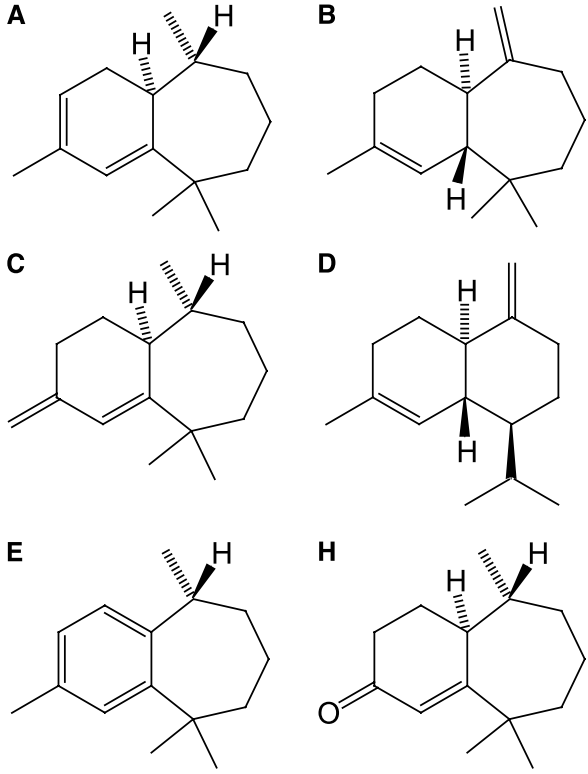

FIG. 1. Male-specific compounds emitted by *P. cruciferae*. Structure lettering corresponds to Bartelt et al. (2001); compounds **F** and **G** in that paper were found in *Aphthona* species but not in *P. cruciferae*. Absolute configurations shown for **A**, **C**, **E**, and **H** were established by chiral synthesis (Muto et al., 2004) and comparison to beetle-derived samples by chiral GC and polarimetry. (**A**) (+)-(6*R*, 7*S*)-2,2,6,10-Tetramethylbicyclo[5.4.0]-undeca-1(11),9-diene; (**B**) (+)-(1*R*,7*R*)-2,2,10-trimethyl-6-methylene-bicyclo[5.4.0]undec-10-ene; (**C**) (+)-(6*R*,7*S*)-2,2,6-trimethyl-10-methylene-bicyclo[5.4.0]-undec-1(11)-ene; (**D**) (+)-γ-cadinene; (**E**) (+)-(*R*)-*ar*-himachalene; (**H**) (+)-(1*S*, 2*R*)-2,6,6-trimethylbicyclo[5.4.0]undec-7-en-9-one.

reported by Pandy and Dev (1968) for the tree compound; thus, both were initially concluded to have the same configuration.

Bartelt et al. (2003) synthesized the racemic forms of the four compounds. Subsequently, Muto et al. (2004) synthesized the individual enantiomers of compounds **A**, **C**, **E**, and **H** using citronellal of known configuration as the chiral starting material. These synthetic studies supported the basic structures and relative stereochemistry reported by Bartelt et al. (2001), but disconcert-

ingly, the absolute configurations determined by Muto et al. (2004) were exactly the reverse of those proposed by Bartelt et al. (2001).

This contradiction was resolved by Mori (2005). The solvent used by Pandy and Dev (1968) to measure the optical rotation of **E** from cedar was chloroform, whereas that used by Bartelt et al. (2001) for beetle-derived **E** was hexane (because several of the beetle-derived compounds encountered in that study deteriorated in chloroform). Mori (2005) discovered, surprisingly, that the optical rotation of **E** in hexane is similar in magnitude V but opposite in sign V to that in chloroform, which explained the previous discrepancy in assigned configurations. Interestingly, compounds **A**, **C**, and **H** do not show a corresponding sign reversal with this solvent change (Bartelt, unpublished data). The structural assignments of Muto et al. (2004), shown in Figure 1, are considered definitive.

Field attractiveness of the sesquiterpenes was first tested by Soroka et al. (2005), using a blend of synthetic racemic **A**, **C**, **E**, and **H** (Bartelt et al., 2003), plus enantiomerically pure **D** (obtained from citronella oil; Soroka et al., 2005). The compounds were formulated on rubber septa so that the emitted ratios of beetle-related enantiomers would be the same as observed in volatile collections from the beetles (Bartelt et al., 2001). Compound **B** was not used in the study because no bulk source was found. Soroka et al. (2005) found that this blend by itself was modestly attractive at two doses, and that the pheromone effect was enhanced when allyl isothiocyanate (allyl ITCN) was present in the trap as well.

In the present paper, we report on the field activity of racemic and enantiomerically pure **A**, **C**, **D**, **E**, and **H** with a European population of *P. cruciferae*, in the presence or absence of allyl ITCN.

METHODS AND MATERIALS

The original objective of this research was to repeat the North American field tests (Soroka et al., 2005) on the European population of *P. cruciferae*. Subsequently, the pure synthetic enantiomers of **A**, **C**, **E**, and **H** became available (Muto et al., 2004), so that it was possible to compare these to the racemic materials. Because the initial field baits also contained **D**, an intermediate field experiment was run to measure its behavioral importance in the initial blend. Then the enantiomers of **A**, **C**, **E**, and **H** were compared directly, all as blends simulating beetle emissions, but lacking **D**. Finally, subtractive tests were conducted to determine whether the components present in minor amounts in the beetle emissions (**C**, **E**, and **H**) were important for attraction. All of the tests included treatments with allyl ITCN.

Electrophysiology. Additional data were acquired during 2003 to evaluate antennal responses of the North American strain of *P. cruciferae* to the six

male-specific compounds. Coupled gas chromatographyYelectroantennographic detection (GC-EAD) was done as described previously (Bartelt et al., 2001). A solution containing the beetle-derived enantiomers of **A**Y**E** and **H** was prepared (ca. 10Y20 ng/μl) and tested with antennae of female beetles (about 1 μl per injection, $N = 4$).

Field Sites. Tests in Hungary were conducted at Budakeszi, Pusztazámor (Pest county), and Nadap (Fejér county). Trapping tests were performed in rapeseed or white mustard fields. Traps were set at the soil level in the weedy edge of the fields. Traps were arranged as blocks so that each block contained one replicate of each treatment. Traps within blocks were separated by 8Y10 m, and blocks were sited 15Y20 m apart.

Capture data were transformed to $(x + 0.5)^{1/2}$ and were analyzed by ANOVA. Treatment means were separated by GamesYHowell test or by BonferroniYDunn test, as appropriate (see also table and figure legends). All

were used with lids closed (so that allyl ITCN penetrated through the walls) in the field tests. Baits were wrapped singly in pieces of aluminum foil and were stored at −65°C until use. In the field, old baits were replaced with new ones at 2- to 3-wk intervals.

Pheromone baits were prepared as described in detail by Soroka et al. (2005). When the standard dose of racemic **A**, **C**, **E**, and **H** was used, the load rates were 500, 34, 56, and 164 μg per septum, respectively. When pure enantiomers of **A**, **C**, **E**, and **H** were used, the weights of the compounds were one half of the racemic amounts (250, 17, 28, and 82 μg per septum, respectively), so that the amount of a given enantiomer was constant. Compound **D**, a single enantiomer, was used at 123 μg per septum. For subtractive blends, the amounts of the components that were included were as above. Low-dose septa were also prepared for experiments 1 and 2, and these had one tenth the amount of material in the standard dose.

Field Experiments. Experiment 1 and 2. These preliminary tests were aimed at studying the activity of the mixture of components **A**, **C**, **E**, and **H** (racemic) and (+)-**D** on its own and the influence of its addition to allyl ITCN. Treatments included traps with components **A**, **C**, **E**, and **H** (racemic) and (+)-**D** at two dose levels, allyl ITCN on its own, its combination with the beetle-related components, and unbaited traps. Polyethylene bag dispensers were used to dispense allyl ITCN. Experiment 1 was run at Nadap, April 1Y18, 2003, with five replicate blocks, in the weedy edge of a field that had been planted in rapeseed in 2002. Traps were inspected twice weekly. Experiment 2 was conducted at Budakalász, March 27YMay 1, 2003, with five replicate blocks, in the weedy edge of a 2002 white mustard field.

Experiment 3. This test was aimed at confirming results of the preliminary tests on the increase of catches when components **A**, **C**, **E**, **H** (racemic), and (+)-**D** and allyl ITCN were presented together in the same trap, but with a lower dose of allyl ITCN (polyethylene vials, see above). Treatments included traps with components **A**, **C**, **E**, **H** (racemic), and (+)-**D** on their own, their combination with allyl ITCN, allyl ITCN on its own, and unbaited controls. The experimental methods were similar to experiments 1 and 2. The site was Budakalász, August 19Y27, 2003, with 10 replicate blocks in a white mustard field (after harvest). Traps were inspected every other day. Allyl ITCN dispensers were replaced with fresh ones on August 23.

Experiments 4 and 5. The objective was to measure the importance of (+)-**D** to the attractiveness of the mixture of beetle-related compounds. Treatments included traps with allyl ITCN on its own (polyethylene vial), its combination with component (+)-**D**, its combination with components **A**, **C**, **E**, and **H** (racemic), or its combination with all components **A**, **C**, **E**, and **H** (racemic) and (+)-**D** and unbaited control traps. These treatments were complemented with traps with a mixture of all components **A**, **C**, **E**, and **H** (racemic) and (+)-**D** on

its own in experiment 5. Two parallel tests were conducted: (1) at Budakalász, August 19Y27, 2003, with 10 replicate blocks in a white mustard field (after harvest); allyl ITCN dispensers were replaced by fresh ones on August 23 (experiment 4); and (2) at Pusztazámor, September 10Y19, 2003 (experiment 5), with six replicate blocks in the weedy edge of a harvested rapeseed field. Allyl ITCN dispensers were replaced by fresh ones on September 15.

Experiment 6. This test was aimed at studying the activity of pure enantiomers of components **A**, **C**, **E**, and **H**. Treatments included traps with allyl ITCN on its own (polyethylene vial), its combination with pure (+) or pure (−) enantiomers of **A**, **C**, **E**, and **H**, or with their racemic mixture, and unbaited control. The test was conducted at Budakalász, September 10Y19, 2003, with 10 replicate blocks in a white mustard field (after harvest). Traps were inspected every other day. Allyl ITCN dispensers were replaced by fresh ones on September 15.

Experiment 7. This test measured whether components **C**, **E**, and **H** could be omitted from the test mixture without apparent loss of activity. A secondary objective was to confirm results in experiment 6 on the activity of pure enantiomers. Treatments included traps with allyl ITCN on its own (polyethylene vial) and traps with allyl ITCN in combination with pure (+), with pure (−), and with racemic **A**, **C**, **E**, and **H**, with only components **A** and **C** (racemic), and with only component **A** (racemic). Traps without bait were also set out as a control. The test was run at Pusztazámor, April 4Y19, 2004, with five replicate blocks in the weedy edge of a white mustard field. Allyl ITCN dispensers were replaced by fresh ones on April 13.

RESULTS

Electrophysiology. In all of the GC-EAD analyses with *P. cruciferae* (example shown in Figure 2), compound **A** was the most strongly detected. Weaker responses were always elicited by **C** and **H** as well, but components **B**, **D**, and **E** elicited no antennal responses.

Field Experiments. Experiments 1, 2, and 3, activity of mixture of components **A**, **C**, **E**, and **H** (racemic) and (+)-**D** on its own, and the influence of its addition to allyl ITCN. In the test at Nadap (Figure 3A), few *P. cruciferae* were caught in traps containing only racemic **A**, **C**, **E**, and **H** and (+)-**D** (referred to below as **ACDEH**), although traps with the larger dose caught more beetles than unbaited traps. More beetles were found in traps with allyl ITCN than in unbaited traps. Catches increased further when dispensers with **ACDEH** were added to traps with allyl ITCN, the difference from addition of allyl ITCN being significant with the larger dose. Catches showed the same trend in the parallel

caught. Traps with the combination of a high dose of allyl ITCN and **ACDEH** caught more than allyl ITCN alone in both tests (Figure 3A, B). Other treatments caught negligible numbers of beetles.

The catches of *P. nodicornis* (Marsham) and *P. ochripes* (Curtis) at Nadap (Figure 3A) and those of *P. nemorum* (Linnaeus) at Budakalász (Figure 3B) also showed similar trends.

In the ensuing tests (experiments 3Ÿ7), the dose of allyl ITCN was decreased in the hope that effects of the **ACDEH** mixture would be easier to detect. In experiment 3, traps with allyl ITCN plus **ACDEH** caught more *P. cruciferae* than allyl ITCN on its own (Figure 4). Both allyl ITCN and the **ACDEH** blend attracted more beetles than unbaited traps, and allyl ITCN on its own attracted more than the **ACDEH** blend. No other flea beetle species were caught in significant numbers in this test.

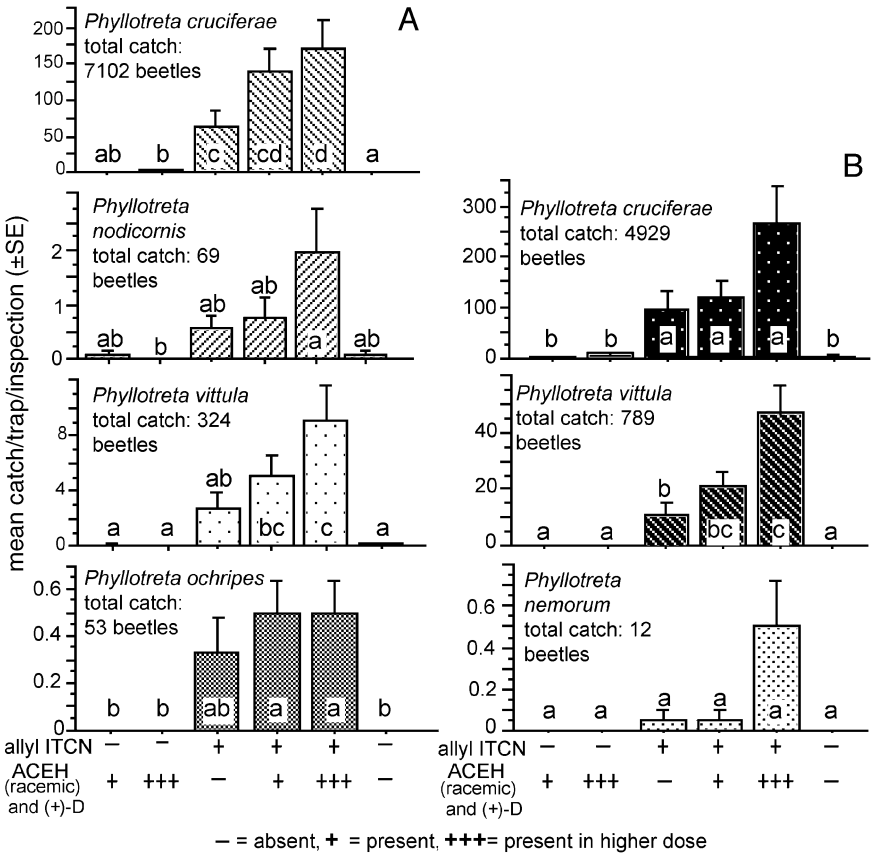

FIG. 3. Catches of flea beetles in traps baited with allyl ITCN, a mixture of racemic **ACEH** and (+)-**D** (at two doses), beetle compounds and allyl ITCN together, or controls. (A) Nadap, April 1Y18, 2003 (experiment 1). (B) Budakalász, March 27YMay 5, 2003 (experiment 2). Significance: within one diagram, bars with the same letters are not significantly different ($P > 0.05$, ANOVA then GamesYHowell). In cases where one or more of the treatments caught no beetles, significant differences from zero catch were checked by BonferroniYDunn ($P > 0.05$).

Experiments 4 and 5, omission of component **D** from the mixture of **A**, **C**, **D**, **E**, and **H**. The largest catches of *P. cruciferae* were recorded in traps with allyl ITCN plus **ACDEH** or **ACEH** [component (+)-**D** subtracted from **ACDEH**], which differed significantly from catches of all other treatments (Figure 5A). Allyl ITCN attracted more beetles than unbaited traps, and allyl ITCN plus component (+)-**D** attracted more beetles than allyl ITCN alone,

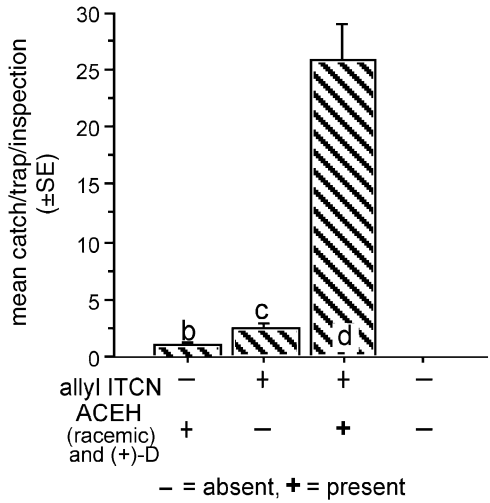

FIG. 4. Catches of *P. cruciferae* in traps baited with allyl ITCN as a single component, a mixture of racemic **ACEH** and (+)-**D**, both baits together, or controls (experiment 3). Budakalász, August 19Y27, 2003. For significance levels, see Figure 3.

suggesting some activity for component (+)-**D**. In this test, *P. vittula* were caught in low numbers, the only treatment catching more beetles than unbaited controls being allyl ITCN plus **ACEH**.

In a supplementary test at Pusztazámor, *P. cruciferae* catches showed similar patterns (Figure 5B), with the exception that traps with the combination of allyl ITCN plus (+)-**D** were no better than allyl ITCN. The mixture of **ACDEH** without allyl ITCN (which was not tested in the previous test in Figure 5B) again showed minimal activity.

In this test, captures of *P. vittula* were best in traps with allyl ITCN plus **ACDEH** or **ACEH** (Figure 5B). Lower catches were observed in traps with allyl ITCN on its own. Unbaited traps or traps with **ACDEH** did not catch beetles.

Similar trends also were observed with *P. nigripes* (Fabricius) (Figure 5B), although no significant difference was found among traps containing allyl ITCN on its own or in combination with other components. Unbaited traps or traps with **ACDEH** alone caught fewer beetles than other treatments.

Experiment 6, activity of pure enantiomers of components **A**, **C**, **E**, and **H**. Blends of the pure enantiomers of **ACEH** with allyl ITCN and racemic **ACEH** with allyl ITCN were equally attractive to *P. cruciferae* (Figure 6). The blend of allyl ITCN with the (−) enantiomers of **ACEH** was no different than allyl ITCN alone.

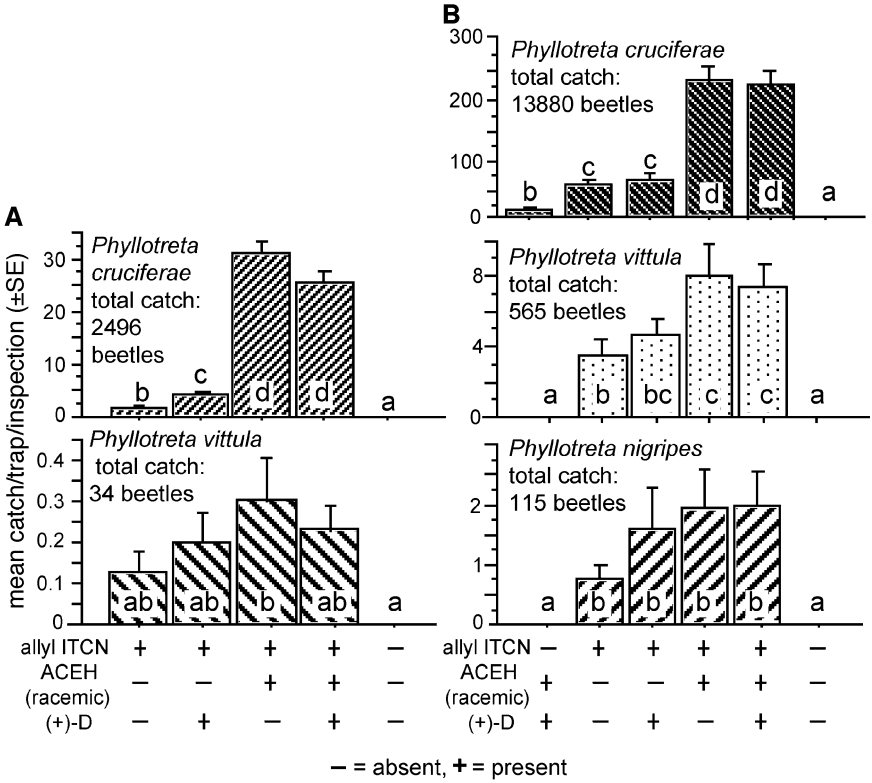

FIG. 5. Catches of *P. cruciferae* in traps baited with allyl ITCN as a single component, or in combination with component (+)-**D**, with racemic mixture **ACEH**, or with all five compounds. (A) Budakalász, August 19Y27, 2003 (experiment 4); (B) Pusztazámor, September 10Y19, 2003 (experiment 5). For significance levels, see Figure 3.

Several other species were caught [*P. vittula*, *P. procera* (Redtenbacher), *P. nigripes*, and *P. undulata* (Kutschera)] in low numbers, but, in general, no treatment was better than allyl ITCN (Figure 6).

Experiment 7, subtraction test of activity of pure enantiomers of components **A**, **C**, **E**, and **H**. More *P. cruciferae* were caught in traps baited with the (+) enantiomers or racemic **ACEH** plus allyl ITCN than in traps with the minus (−) enantiomers or with allyl ITCN on its own (Figure 7). Activity did not decrease when components **E**, **H**, and **C** were removed from the blends, indicating minimal or no role of **E**, **H**, or **C** as pheromone components.

Catches of *P. vittula* showed the same tendency, but the only treatment attracting more than allyl ITCN on its own was the racemic **ACEH** plus allyl ITCN combination (Figure 7).

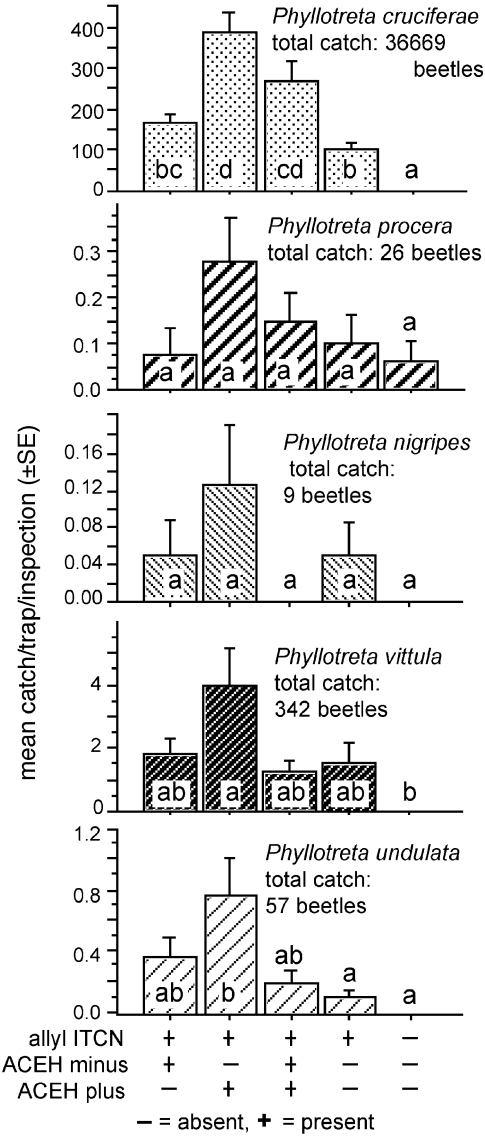

FIG. 6. Catches of *Phyllotreta* species in traps baited with allyl ITCN or its combination with (+) or (−) enantiomers of **ACEH**, or racemic **ACEH**. Budakalász, September 10Y19, 2003 (experiment 6). For significance levels, see Figure 3.

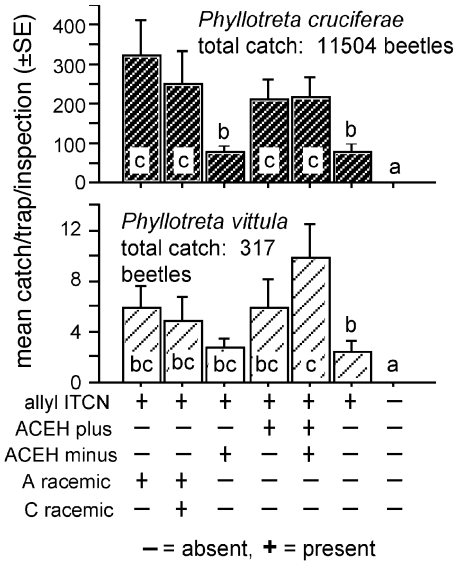

FIG. 7. Catches of *Phyllotreta* species in traps baited with allyl ITCN or its combination with (+) or (−) enantiomers of **ACEH** or with racemic **ACEH**, **AC**, or **A**. Pusztazámor, April 4Y19, 2004 (experiment 7). For significance levels, see Figure 3.

DISCUSSION

In the present study, a mixture of components **A**, **C**, **D**, **E**, and **H**, although marginally attractive on its own, increased catches of *P. cruciferae* when presented together with the host-plant-derived attractant allyl ITCN. This is consistent with the results from North America (Soroka et al., 2005), suggesting that the pheromone compositions of the European and North American populations of *P. cruciferae* are similar.

There are a number of examples known of insect pheromones being synergized by host volatiles. In the well-documented case of *Melolontha* scarab beetles (Coleoptera, Scarabaeidae), males orient towards green leaf volatiles originating from damaged leaves from feeding female beetles (Ruther et al., 2000, 2002). Green leaf volatiles are somewhat attractive (Imrei and Tóth, 2002; Reinecke et al., 2002b), but many more beetles are attracted to blends of the green leaf volatiles with the pheromone components benzoquinone or toluquinone (Reinecke et al., 2002a; Ruther and Hilker, 2003). The quinones on their own show no activity. Similarly, with the scarab *Oryctes elegans* Prell. (Coleoptera, Scarabaeidae), the main aggregation pheromone component alone

was minimally attractive but, when presented together with host plant odor, it was clearly synergistic (Rochat et al., 2004).

Similar cases of strong synergism between pheromone components and host volatiles have been described for several species of *Rhynchophorus* weevils (Coleoptera, Curculionidae, Jaffé et al., 1993; Giblin-Davis et al., 1994; Oehlschlager et al., 1995; Rochat et al., 1995) and *Carpophilus* spp. sap beetles (Coleoptera, Nitidulidae; reviewed by Bartelt, 1999).

In the present study, beetles of both sexes were caught, but no special effort was made to study possible sex-specific differences in responses of *P. cruciferae*. In any case, effects would have been obscured by the activity of allyl ITCN, which attracts both sexes. It remains to be determined whether the communication strategy of *P. cruciferae* is sex-related, as in *Melolontha* scarabs, or is of the aggregation type as in the other examples above.

The biological activity of the insect-produced compounds was clearly connected with chirality. Because the (−) enantiomers were not inhibitory, racemic samples could be used as bait components in trapping studies. Furthermore, compounds **C**, **D**, **E**, and **H** had no obvious biological activity. Compound (+)-**A** [(6*R*,7*S*)-2,2,6,10-tetramethylbicyclo[5.4.0.]undeca-9,11-diene] was the only male-specific compound for which pheromonal activity could be clearly established for *P. cruciferae*. The closely related *P. vittula* responded in similar fashion to *P. cruciferae* with respect to the component mixtures and pure enantiomers tested. This suggests that compound **A** plays a role in pheromonal communication of this species as well. It remains to be seen whether *P. vittula* males produce this compound. *P. vittula* is among the economically most important pest flea beetles in Europe (Kaszab, 1962; Jourdheuil, 1966; Vig, 1996). Other *Phyllotreta* species were caught in low numbers, and the general trends resembled those seen with *P. cruciferae* and *P. vittula*. This suggests that the compounds tested may occur widely within the genus. Previously, compound **A** was found to be a common major component in the emissions collected from males of several North American flea beetle species (Bartelt et al., 2001) but at present, field activity data are available only for *P. cruciferae*.

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